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CHARACTERIZATION OF THYMOQUINONE /HYDROXYPROPYL- β -CYCLODEXTRIN INCLUSION COMPLEX: APPLICATION TO ANTI-ALLERGY PROPERTIES

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Abstract:

Thymoquinone is an effective phytochemical compound in the treatment of various diseases. However, its practical administration has been limited due to poor aqueous solubility and bioavailability. In this work, we developed a novel inclusion complex of thymoquinone and hydroxypropyl- β -cyclodextrin that features improved solubility and bioactivity. The drug solubility was markedly accelerated in the increasing ratio of hydroxypropyl- β -cyclodextrin to thymoquinone amount. The formation of the thymoquinone/ hydroxypropyl- β -cyclodextrin inclusion complex was evidenced using X- ray diffraction, differential scanning calorimetry, thermal gravimetric analysis, Fourier transform infrared, scanning electron microscopy and nuclear magnetic resonance. The release behavior of the complex, as well as of their mixtures, was examined in artificial gastric (pH 1.2) and intestinal (pH 6.8) dissolution media. The formulated complex released the drug rapidly at the initial stage, followed by a slow release. Thermodynamic parameters ΔH , ΔS and ΔG were calculated with temperatures ranging from 20 to 45 $^{\circ}\text{C}$ to evaluate the complexation process. The activity of the inclusion complex was evaluated on IgE-mediated allergic response in rat basophilic leukemia (RBL-2H3) cells by monitoring key allergic mediators. The results revealed that compared with free thymoquinone, the inclusion complex more strongly inhibited the production of the release of histamine, tumor necrosis factor- α , and interleukin-4, and was not cytotoxic at the tested thymoquinone concentrations (0.125–4 $\mu\text{g/mL}$) indicating the inclusion complex possibly had better antiallergic effects. Our finding suggested that the inclusion complex achieved prolonged action and reduced side-effect of thymoquinone.

Thymoquinone; Hydroxypropyl- β -cyclodextrin, Solubility; Thermodynamic parameters; Drug release; Inclusion complex; *In vitro* anti- allergy; Physicochemical characterization

1 Introduction

Nigella sativa, known *habbat albarakah* (Arabic “seed of blessing”) among Arabs and Malays (Naz, 2011) has long been traditionally used for the treatment of fever, headache, anxiety, diarrhea, asthma, stroke, and allergies (Aziz et al., 2011; Duncker et al., 2012; Işık et al., 2010; Salem, 2005).

Thymoquinone (TQ) is the phytochemical that gives the yellow color to the black seed oil (Magdy et al., 2012; Zavarin and ANDERSON, 1955) (Figure 1). TQ is the primary compound in *Nigella sativa* that was shown to have several pharmacological activities (Badary et al., 2003; Gali-Muhtasib et al., 2006; Tekeoglu et al., 2007) including anticancer (H El-Far, 2015), anti-viral (Umar et al., 2016), gastroprotective (Magdy et al., 2012), anti-diabetic (Fararh et al., 2005; Pari and Sankaranarayanan, 2009), antimalarial (Johnson–Ajinwo et al., 2018), and antimicrobial (Harzallah et al., 2011) properties.

Aziz et al. (2011) investigated the inhibitory effect of TQ on histamine release from sensitized mast cells. After sensitization by a mixture containing egg albumin and attenuated bacilli, rats were intraperitoneally administered with TQ at 8 mg/kg (Aziz et al., 2011). Rat peritoneal mast cells (RPMCs) were collected from male albino rats to quantify the histamine released from the immune cells. A significant activity was detected at dosing of 8 mg/kg TQ. Moreover, the TQ exhibited a protective activity to vital organs in a dose-dependent manner (Aziz et al., 2011).

Among the advantages of the nanoparticulated drug delivery system is that it can be administered several routes including intravenous injections (Al-Qubaisi et al., 2013a; Al-Qubaisi et al., 2013b). Because of the versatility of this drug carrier system, administration of drugs by intravenous injections (Chrastina et al., 2011; Lindqvist and biovetenskap, 2015; Mirtič et al., 2015; Phillips et al., 2010). Cyclodextrins are ideal drug carriers because they are not only water soluble but also possess a structure with nanocavity that could incorporate therapeutic compounds (Brewster and Loftsson, 2007). The hydrophobicity of TQ that affects bioavailability poses a challenge for the development of this compound into a drug (Martinovich et al., 2016). Poorly soluble compounds like TQ are poorly absorbed in the gastrointestinal tract and do not readily enter blood circulation to elicit their actions (Odeh et al., 2012). One of the means to improve solubility of these compounds is to incorporate in a drug carrier, such as 2-hydroxypropyl β -cyclodextrin (HP β CD) (Gad, 2008).

The famous monoaminopropyl compound, chlorpheniramine, which showed potent antihistaminic activities, was developed followed by brompheniramine. Chlorpheniramine is a highly active, strong antihistamine with serious side-effects that represents a threat to life of patients at high doses. The encapsulation of chlorpheniramine by different types of cyclodextrin produced complexes of long-lasting anti-allergic activity and offered good protection at lower doses than encapsulated compounds (Tang and Ng, 2008).

HP β CD is a modified derivative, an alternative to α , β and γ -cyclodextrin, of high water-solubility and safe for parenteral applications (Mura et al., 2001; Peeters et al., 2002). HP β CD has a hydrophobic cavity of size ranging 0.6 to 0.65 nm in diameter (Manasco et al., 2012). The compound is infinitely soluble in water at room temperature. It has great ability for complexation with other compounds. Its safe biological profile permits for wider application with a variety of routes of administration (Acton, 2012; Amiri, 2017; Muzumdar and Pharmacy, 1999).

In this study, thymoquinone-loaded hydroxypropyl- β -cyclodextrin (TQ/HP β CD) was formulated and characterized physicochemically. The anti-allergic reaction of TQ/HP β CD inclusion complex was examined to determine its therapeutic potential.

2 Materials and Methods

2.1 Chemicals and reagents

Trypsin/ethylenediamine tetracetic acid (EDTA) solution was purchased from Invitrogen (Carlsbad CA, USA). Dimethylsulfoxide (DMSO), phosphate-buffered saline (PBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dulbecco's modified Eagle's medium (DMEM) and trypan blue dye were purchased from Sigma Chemical Company.

(Perth, WA, Australia). HP β CD (MW 1396) and TQ (MW 164) were purchased from sigma Aldrich (St.louis, MO, USA). Average degree of substitution in HP β CD is 0.67 hydroxypropyl groups per glucose unit.

2.2 Preparation of TQ/HP β CD complex and the physical mixture

The TQ/HP β CD complex were prepared by dissolving 1.396 g of HP β CD and 0.164 g of TQ in 100 ml of deionized water to obtain 1:1 molar ratio solution. The solution was incubated in an ultrasonic bath for 2 h and then maintained under agitation for 3 days at room temperature. The solution was filtered through 0.45 μ m filter and the clear solution was frozen at -80°C , and subsequently freeze-dried for 24 h at -55°C and then ground using mixing bowl to get powder. The physical mixture of TQ and HP β CD in the same weight ratio as the dried complex was prepared. The TQ and HP β CD were admixed in a mortar and pestle for 5 min to obtain a homogenous powder.

2.2.1 Phase solubility

To determine phase solubility, 5 mL vials containing aqueous solutions of different concentrations of HP β CD (0 to 0.01 mM) were mixed with excess amounts of TQ (up to 150 mg) and shaken at) at $20-45^{\circ}\text{C}$ for 72 hrs. The samples were then filtered through 0.45 μ m nylon filter. The concentration of TQ was measured at preset time intervals with $\lambda_{\text{max}} = 254$ nm using UV-Vis spectrophotometer (Model Perkin Elmer Lambda 35).

The apparent stability constant K was calculated from the phase solubility diagram using the formula:

$$K = \frac{\text{slope}}{S_0 (1 - \text{slope})} \quad (1)$$

where S_0 (the intercept) denotes the solubility of TQ in the absence of HP β CD and slope is the slope of the linear part of all the solubility curves (Labandeira and Vila-Jato, 2012).

The change in entropy (ΔS) is given by the following equation;

$$\ln K = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (2)$$

where ΔH refers to the changes in enthalpy, R is the universal gas constant (8.314 J/mol K) and T is the experimental operating temperature (293, 303, 310, or 318 K).

ΔG (Gibbs' free energy) is defined through the following mathematical equation;

$$\Delta G = \Delta H - T\Delta S \quad (3)$$

2.3 Physicochemical Properties of TQ/HP β CD inclusion complex

2.3.1 X-ray Diffraction

Powder X-ray diffraction (XRD) patterns of powdered complex were recorded with a Shimadzu XRD-6000 instrument (Shimadzu Corporation, Kyoto, Japan) in the range of 10° – 70° using $\text{CuK}\alpha$ as a radiation source ($\lambda = 1.5418 \text{ \AA}$) generated at 30 kV and 30 mA.

2.3.2 Thermogravimetric analysis

Thermogravimetric and differential thermogravimetric analyses (TGA-DTG) were recorded on a Mettler Toledo instrument with a heating rate of 10 °C/min under a nitrogen atmosphere (N₂ flow rate 50 mL/min).

2.3.3 Fourier transform infrared

Fourier transform infrared (FTIR) spectra for powdered complex were recorded over the range of 400–4000 cm⁻¹ on a Thermo Nicolet Nexus, Smart Orbit spectrometer using a sample of approximately 1% in 200 mg of spectroscopic-grade potassium bromide (KBr) with 10 tons of pressure.

2.3.4 Differential scanning calorimetry

Differential scanning calorimetry (DSC Model Mettler Toledo 821^e) was used to identify the sample's melting point (T_m) in the temperature range of (23 to 599 °C) using nitrogen gas pressure at a heating rate of 10 °C/min.

2.3.5 Nuclear magnetic resonance spectroscopy

The NMR spectra were recorded on a Bruker AMX-400 NMR spectrometer equipped with a dedicated 5 mm ¹H probe at 25 °C, operating at 400.14 MHz. Two-dimensional phase-sensitive Nuclear Overhauser effect spectroscopy (NOESY) was achieved using time proportion phase incrementation (TPPI) (II), and magnitude homonuclear correlation spectroscopy (COSY) was obtained in the usual way. 400 t_1 values were used, and free induction decays in t_2 were recorded in 1024 complex-point blocks, summing either 512 (COSY) or 1024 acquisitions (NOESY) per t_1 . The NOESY mixing time was 30 ms. Solvent suppression performed by saturation during the relaxation delay (300 ms); the 90° transmitter pulse was - 10 p.s. The data sets were zero filled to 1024 x 1024 data point sets after digital filtering with a 30° to 45° (NOESY) or 0° (COSY) phase-shifted sine-bell-squared function in both dimensions. Chemical shifts for all ¹H spectra were referenced to sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) through the residual water resonance at 4.76 ppm. Spectra were processed on the spectrometer-based X-32 computer, using the manufacturer's software package, LIXNMR. The ¹H NMR experiments were obtained in deuterated methanol on JEOL ECA -400 (400MHz) spectrometer at room temperature. Chemical shifts are given in ppm relative to tetramethylsilane as an internal standard ($\delta = 0$).

2.3.6 Hydrodynamic size and zeta potential

The hydrodynamic size and zeta potential of powdered complex dispersion (1 µg of sample dispersed in 1 mL of ultraionized water) were characterized using a ZetaSizer Nano ZS (Malvern Instruments Ltd Malvern, UK) with dynamic light scattering.

2.3.7 Scanning electron microscopy

Scanning electron microscopy (SEM, Model LEO 1450VP [LEO Electron Microscopy Ltd Cambridge, UK]), with an accelerating voltage of 30 kV, was used in order to investigate the morphology of powdered complex. The sample was degassed in an evacuated heated

chamber at 100°C overnight. Prior to SEM scanning, dried samples were spread over double-sided conductive tape which was adhered to a specimen stub.

2.4 Drug loading and release

2.4.1 Drug loading

We made a calibration curve for a standard and then calculate the drug loading and drug entrapment efficiency using the following equations:

The entrapment efficiency % = (initial drug amount in formulation (mg)- untrapped drug (mg)) x 100 / initial drug amount in formulation (mg) (4)

The drug loading = (initial drug amount in formulation (mg)- untrapped drug (mg)) x 100 / complex amount (mg) (5)

2.4.2 Drug release

In vitro release of TQ from complex in stomach and intestine was determined by adding approximately 1.0 mg of complex in 3 mL of either artificial gastric juice [pH 1.2, phosphate buffered saline solution (PBS) containing 0.1 μM HCl and 3.2 % Pepsin], or artificial intestinal juice (: pH 6.8, PBS containing 3 μM phospholipid and 10 μM bile salt). The cumulative amount of TQ released into the solution was measured at preset time intervals with $\lambda_{\max} = 254$ nm using UV-Vis spectrophotometer (Model Perkin Elmer Lambda 35). To analyze the profile of drug release from TQ/HPβCD inclusion complex, software KinetDS 3.0 (Mendyk *et al.*, 2012) was used. The results of drug release were fitted to kinetic models of zero-order (6) and first-order (7), second-order (8), Higuchi (9), Korsmeyer Peppas (10), Weibull (11), Hixson-Crowell (12), Michaelis–Menten (13) and Hill (14). The best-fit determines the model that best described the drug release curve, mainly by the coefficient of determination R^2 , empirical coefficient of determination R^2_{emp} (19), Akaike information criterion AIK (20), Bayesian information criterion Schwarz criterion BIC (21) and Root-mean-squared error RMSE (22). The dissolution efficiency DE% (15) and mean dissolution time MDT (16) have been also computed by KinetDS 3.0 software (Mendyk *et al.*, 2012).

$$Q = k \cdot t + Q_0 \quad (6)$$

$$\frac{1}{Q} = k \cdot t + \frac{1}{Q_0} \quad (7)$$

$$\frac{1}{Q^2} = k \cdot t + \frac{1}{Q_0^2} \quad (8)$$

$$Q = k \cdot \sqrt{t} \quad (9)$$

$$Q = k \cdot t^n \quad (10)$$

$$Q = 100 \cdot \left[1 - \exp \left(\frac{-(t - T_{LAG})^b}{a} \right) \right] \quad (11)$$

$$Q^{\frac{1}{3}} = k. (t - T_{LAG}) + Q_0^{\frac{1}{3}} \quad (12)$$

$$Q = \frac{Q_{max}.t}{k+t} \quad (13)$$

$$Q = \frac{Q_{max}.t^n}{k^n+t^n} \quad (14)$$

$$DE = \frac{\int_0^t Q dt}{Q_{max}.t} * 100 \quad (15)$$

$$MDT = \frac{\sum_{j=1}^n t_j^{AV} * \Delta Q_j}{\sum_{j=1}^n \Delta Q_j} \quad (16)$$

$$\Delta Q = Q_{(t)} - Q_{(t-1)} \quad (17)$$

$$t_j^{AV} = (t_i + t_{i-1})/2 \quad (18)$$

$$R_{emp}^2 = 1 - \frac{\sum_{i=1}^N (y_i - \hat{y}_i)^2}{\sum_{i=1}^N (y_i - y_{AV})^2} \quad (19)$$

$$AIC = 2k + N. [In(\sum_{i=1}^N (y_i - \hat{y}_i)^2)] \quad (20)$$

$$BIC = N. In(\sum_{i=1}^N (y_i - \hat{y}_i)^2) + k. In(N) \quad (21)$$

$$RMSE = \sqrt{\frac{(\sum_{i=1}^N (y_i - \hat{y}_i)^2)}{N}} \quad (22)$$

Q: amount (%) of drug substance released at the time t Q₀: start value of Q Q_{max}: maximum value of Q (100%) T: time k, a, b: constants T_{LAG}: lag time y_i : observed value $\hat{y}_i$: model-predicted value y_{AV}: average output value (Patel et al., 2008).

2.5 Cells culture

Rat basophilic cells (RBL-2H3) were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA), and characterized as virus negative. They grow as an adherent monolayer of tightly knit epithelial cells.

2.6 In vitro Anti-allergic of TQ/HPβCD

2.6.1 Cytotoxicity MTT assay

The RBL-2H3 cells lines were plated at 2×10^4 cells/well by adding 200 μL of 1×10^5 cells/mL suspension to each well of a 96-well tissue culture plate. The plates were incubated for a two days to ensure attachment at 70 to 80% confluency. The media was aspirated and replaced with 200 μL fresh media containing TQ either encapsulated in HPβCD complex of

different concentrations (0.125 to 4.0 $\mu\text{g/mL}$), or dissolved in DMSO (0.125 to 4.0 $\mu\text{g/mL}$) and pure HP β CD (0.125 to 4.0 $\mu\text{g/mL}$). The last row was left as an untreated control. The plates were incubated at 37 °C, 5% CO₂, for 24 h. After incubation, the media was aspirated and the cells washed with PBS buffer 3 times to ensure that all compounds have been removed. 200 μL of fresh media was added to each well. 20 μL MTT solution made to a total of 200 μL with medium was added to each well, mixed gently, and incubated for 4 to 6 h at 37 °C under 5% CO₂. The MTT-containing medium was then carefully removed and replaced with 200 μL DMSO/well to dissolve the formazin crystals. The plates were read on an automated spectrophotometric EL 340 multiplate reader (Bio-Tek Instruments Inc., USA) at 570 nm.

2.6.2 Histamine, IL-4 and TNF- α release

The wells of 24-well tissue plate were seeded with 0.5 μL 1×10^6 RBL-2H3 cells/well. The plate was incubated overnight at 37°C in a 5% CO₂ atmosphere to assure attachment and 90% confluency. The media was aspirated and replaced with 500 μL fresh medium containing 0.2 $\mu\text{g/mL}$ of IgE (MP Biomedicals) and the plate incubated for 1 h. The medium was aspirated and washed with release buffer containing 1.25 mg/mL anti-IgE and different concentrations (0.125 to 4.0 $\mu\text{g/mL}$) of TQ dissolved in DMSO or complexed with TQ-HP β CD complex before incubating with shaking at 37°C for 10 min. Cetirizine was used as positive control for histamine release. Released histamine was determined using a histamine RIA kit (MP Biomedicals). The IL-4 and TNF- α levels in the supernatant were determined using the rat IL-4 and TNF- α ELISA kits (R&D Systems).

2.7 Statistical analysis

All experiments were done in triplicates unless otherwise indicated. Data were expressed as means $\pm$ standard deviation. All statistical analyses were performed using the Minitab statistical software (Minitab Inc., State College, PA). The significance of treatment effects were determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests. A value of $p < 0.05$ was considered significant unless otherwise indicated.

3 Results and Discussion

3.1 Physicochemical Characterization

3.1.1 X-Ray diffraction analysis

The XRD patterns of TQ, powdered TQ-HP β CD complex, HP β CD and physical mixture are shown in Figure 2. The diffractogram of TQ exhibited a series of intense peaks showing that this compound is crystalline in nature. The peak at about $2\theta = 10.4^\circ$ correspond to TQ. The diffractogram for HP β CD was devoid of a peak, showing that it is amorphous. The physical mixture shows a clear peak corresponding to TQ. TQ/HP β CD inclusion complex similar to HP β CD did not show any peak indicating successful complex formation. Thus, the XRD analysis showed that incorporation of TQ in HP β CD eliminates the tendency for TQ to form crystals. The TQ/HP β CD inclusion complex is a solid amorphous powder. The XRD analysis also showed that TQ is well-dispersed in the complex.

3.1.2 Fourier transform infrared

The FTIR for TQ, HP β CD, TQ-HP β CD, and physical mixture are shown in Figure 3. The band at 2970 cm^{-1} resulted from CH_2 stretching vibration. An intense sharp band at 1647 cm^{-1} is due to the carbonyl stretching mode. The bands at 1457 and 1384 cm^{-1} are due to C=C bending vibration (aliphatic). The bands at 1245, 1157, 1083, and 1034 cm^{-1} are owing to a C–O–C stretching vibration. The interaction between TQ and HP β CD resulted in the disappearance of these bands. Under FT-IR analysis of the TQ/HP β CD inclusion complex shows that the stretching bands were either shifted, entrapped or disappeared, indicating that there is complete interaction between TQ and HP β CD, a finding similar to that reported earlier (Srinivasan et al., 2011).

3.1.3 Differential scanning calorimetry

The DSC curves for TQ, HP β CD, TQ/HP β CD inclusion complex, and physical mixture are shown Figure 4. Pure TQ gives rise to a sharp peak that corresponds to melting at 48.44 °C. The HP β CD showed two peaks at 72 and 158 °C, which correspond to glass transition (T_g) and melting point (T_m) temperatures, respectively. By physically mixing TQ with HP β CD, the T_g and T_m values were at approximately 52, and 152 °C, are respectively lower the values shown by HP β CD. The DSC pattern for TQ/HP β CD inclusion complex is similar to that for HP β CD. With lower T_g and T_m values in TQ/HP β CD inclusion complex than HP β CD shows that there is thermal stability in the inclusion complex. Unlike the TQ and HP β CD physically mixed sample, the thermal stability of TQ/HP β CD inclusion complex decreased. The T_g (glass transition) of TQ/HP β CD inclusion complex was lower than that of HP β CD. This indicates that the complex showed less tendency to crystallize and maintaining a carrier system that facilitates release of the loaded TQ. This is a desired property of drug delivery system.

3.1.4 Thermal gravimetric analysis

The thermal gravimetric analysis thermogram (TGA-DTG) for TQ, HP β CD, and TQ/HP β CD inclusion complex samples are illustrated in Figure 5. It is apparent by the thermograms that there is a 2-step degradation process. The first dip in the differential TGA thermogram corresponds to the minimum weight loss during the initial degradation temperature of 55 °C, which is ascribed to trapped moisture in the HP β CD powder. The second dip at 354 °C refers to the maximum weight loss attributed to the major degradation of HP β CD sample. At 401 °C, HP β CD sample is converted to carbonaceous residues, thus percentage weight loss change becomes independent with increasing temperature.

For TQ/HP β CD inclusion complex sample, the thermogram followed the same trend exhibited by the HP β CD. With the introduction of TQ on the surfaces of powdered complex, the initial degradation temperature and the maximum weight loss temperature becomes 54 and 344 °C, respectively. The complexation sample was observed to be eliminated at a temperature of 392 °C, above which insignificant weight loss change occurred with increase in temperature.

3.1.5 Nuclear magnetic resonance spectroscopy

3.1.5.1 One-dimensional (1D) ^1H NMR

The NMR experiment has been performed using deuterated water (D_2O) with shaking, the OH proton is rapidly exchanged for deuterium (D) and the OH becomes OD, disappearing from the ^1H spectrum. Figure 6 shows the monodimensional ^1H NMR for TQ, HP β CD, and TQ/HP β CD inclusion complex samples. The resonance peaks of TQ, including the NMR peak at the chemical shift (δ) of 1.10 ppm, corresponding to the protons (Ha) of the CH_3 groups of the side chain in the TQ molecule, 1.99 ppm, corresponding to the protons (Hb) of the methyl group in the meta position of the TQ aromatic ring, are clearly evident. The methane group protons (Hc) showed NMR resonance at (δ) of 2.9 ppm, while the protons (Hd) displayed the characteristic NMR peaks of the aromatic rings, at (δ) of 6.5 and 6.6 ppm. The HP β CD protons are labeled as H1-H6, representing the protons of the cyclodextrin (CD) rings, and the NMR peaks (Table 1).

The changes in the chemical shifts of HP β CD protons, calculated as the difference in the chemical shift ($\Delta\delta$), in the absence and presence of the TQ in the cavity of the CD are shown in Table 1. There was a downfield shift (H3 and H5) of the inner protons in the HP β CD cavity and the protons (H6) of inner side of the CD molecule (Figure 7).

These results indicate that the TQ-HPBCD inclusion complex was obtained successfully. On the other hand, the outside protons H1, H2, and H4 showed a clear up-field shift, confirming that the H3 and H5 protons were more affected by the guest molecule, and the H3 protons were more affected due to their proximity to the guest moieties, included inside the cavity, suggesting that the TQ was included from the wide rim (hydrophobic side) of the CD molecules. In addition, the protons of the CH_3 (Ha) of the side chain of the TQ, and the (Hc) proton of the adjacent methane groups, showed downfield shifting. These results imply that the TQ was incorporated in the cavity of HP β CD molecule.

3.1.5.2 Two-dimensional (2D) NMR Analysis

The 2D NMR Correlation Spectroscopy (COSY) and Nuclear Overhauser Effect Spectroscopy (NOESY) experiments were also performed to further characterize the inclusion complex.

3.1.5.2.1 2D NMR Correlation Spectroscopy (COSY)

Figure 8 shows the 2D ^1H - ^1H COSY spectrum of TQ-HPBCD complex. There were no correlations observed between the aromatic protons of the TQ rings (Hd) and the CD protons (H1-H6), suggesting that the aromatic ring is still free and no interaction between these protons, and thus the ring is located outside the cavity of the CD molecules. On the other hand, there are cross-peaks between the protons (Ha) of the CH_3 groups in the TQ side-chain that resonated at the chemical shift of 1.14 ppm, and the inner protons of the HP β CD (H3). The results show that the methyl protons are directly attached to the H3 of the HP β CD molecules.

3.1.5.2.2 2D NMR NOESY Spectrum

The 2D NMR NOESY spectrum of the TQ/HP β CD inclusion complex was obtained and shown in Figure 9. NOESY spectrum showed an obvious cross-peak interaction between TQ hydrocarbon side-chain (Ha) with the H3 and H5 internal protons of the cyclodextrin. In

addition, there is an interaction observed between the Ha (CH₃ of TQ) and H6 protons of HPβCD cavity. This interaction indicates penetration of the aliphatic tail (side-chain) into the HPβCD cavity. There are no detectable interactions between the aromatic protons of the TQ ring (Hd) or the methyl group protons (Hb) at the meta position of the TQ ring with the HPβCD internal protons, suggesting that the TQ ring is located within the cavity of the CD molecule (see Figure 7).

Consequently, the ¹H NMR spectrum showed chemical shift changes upon the formation of the TQ-HPβCD complex, a finding similar to that reported earlier (Fernandes et al., 2003; Torri et al., 2007; Tóth et al., 2013). This was confirmed by the 2D COSY analysis that showed that the hydrocarbon side chains of the TQ molecules completely included inside the HPβCD cavity. These are further evidence of the successful incorporation of TQ in HPβCD to form a potential nanoparticulated carrier therapeutic compound. The nuclear overhauser effect (NOE)-based experiment was carried out to investigate the inclusion complex structure and the spatial proximity between the atoms of the host and the guest by observing the intermolecular dipolar cross-correlations. The closely located protons in the space can produce a NOE cross-interactions, and the NOESY spectrum displays a cross-correlation between protons of different species if they are close with spatial contact within 0.4 nm (Yang et al., 2009). It is well known that the inclusion of the guest molecule inside the cavity of the cyclodextrin cavity induces changes in the chemical shift of the guest protons, and the affected protons of the host (Pose-Vilarnovo et al., 2001).

3.1.6 Drug loading and release

The drug-loading and encapsulation efficiency of TQ in the TQ/HPβCD inclusion complex were 10.51 and 99.993%, respectively. The rapid initial release of TQ from the complex in artificial gastric and artificial intestinal juices is due to weak adsorption or binding of to the surface of the HPβCD. The results suggest that TQ was not satisfactorily incorporated into HPβCD cavity (Figure 10). A much slower sustained release of TQ (~75%) from TQ-HPβCD in artificial intestinal juice (pH 6.8) over a prolonged period of 42 h than in artificial gastric juice (pH 1.2). The cumulative release of TQ in artificial gastric juice was more than 50% within 9 hours, while that in intestinal juice it was less than 40%. The dissolution media used on the compounds in this study was based on the physiological environment fasted-state in the stomach and small intestine, including pH and ion and bile salts concentration (Jantratid et al., 2008). The TQ/HPβCD inclusion complex was in powder form to formulate as sustained-release tablet, to enhance solubility of TQ with pH favoring orally application of the compound (Mahato and Narang, 2017). In this study, the mean dissolution, or drug release of TQ/HPβCD inclusion complex in artificial gastric juice was almost complete (98%) after approximately 27 h. In artificial gastric and intestinal juices, dissolution of the complex reached 85% after 18 and 100 h, respectively. The different release rates at both artificial juices are possibly due to the different release mechanism for TQ from the organic host (inclusion complex). At a high acidic media, inclusion complex may be easily dissolved and the release of TQ from the HPβCD cavity might occur due to the breakdown of van der Waals or hydrophobic types of interaction in which the hydrophobic guest (TQ) has penetrated through HPβCD together with the ion exchange process. On the other hand, at artificial intestinal juices, inclusion complex are

more stable, and as a result, release would occur through an anion exchange process. The Food and Drug Administration stipulated that the release of an encapsulated drug in a dissolution test must be not less than 85% of within 30 min (Shabir, 2003).

3.1.6.1 Release kinetics of TQ from TQ/HP β CD complex

To analyze the profile of drug release from prepared complex, software KinetDS 3.0 (Mendyk et al., 2012) was used. The results of drug release were fitted to different kinetic models as seen in Figures 11 and 12. The readings were fitted to nine models describing drug dissolution. The AIK, BIC, R^2_{emp} , RSME values were the best for describing the release kinetics of TQ from the TQ/HP β CD complex (Table 2 and 3). In artificial gastric and artificial intestinal juices, the release profile of TQ did not follow the zero order, first order, Hixson-Crowell, or Higuchi kinetics. The kinetic model that best described the dissolution curves for all formulations was the Weibull model, with lowest reading observed at R^2_{emp} , RMSE, AIC and BIC while R^2 did not predict best model.

The Weibull model is very suitable for evaluate the kinetic release from matrix type drug delivery MTDD. Weibull model kinetics enables the fast assessment and quantification of proportionality, or lack thereof. Moreover, this model allow the pharmacists and clinicians to predict how data might mature over time, something that is of great interest in disease treatments (Ramteke et al., 2014).

3.1.7 Phase solubility

Figure 13 demonstrates the phase solubility of TQ in presence of HP β CD at various temperatures. It is apparent that the solubility of TQ increases linearly with increase in molar ratio of HP β CD.

3.1.8 Thermodynamic analysis

The enthalpy change (ΔH), the entropy change (ΔS) and the Gibbs free energy change (ΔG) are shown in Table 4 after calculating the relationship between solubility and temperature (Figure 14). The negative values for the change in enthalpy under various temperatures show that the interaction that took place between TQ and HP β CD was exothermic. The high negative ΔH values could be explained based on two perspectives; first, it is an indication of strong interaction occurring between TQ and HP β CD that could be due to van der Waals or hydrophobic types of interaction in which the hydrophobic guest (TQ) has penetrated through or dehydrated in HP β CD sample. Second, the higher negative values of ΔH imply higher degrees of stabilization attained in the TQ/HP β CD inclusion complex as due to the fact that the change of enthalpy of any inclusion process is largely dependent on the stabilization of the complexation system (Eid et al., 2011).

The change in entropy (ΔS) also tends to have negative values for the inclusion process of TQ into HP β CD cavity. It is well known that ΔS depends largely on the degrees of freedom of translational, vibrational and rotational motions of the molecules which is given by the following equation;

$$\Delta S = k \ln W \quad (23)$$

where, W is the number of microstates corresponding to the degrees of freedom of translational, vibrational and rotational motions of the molecules, k is the Boltzmann constant, 1.38×10^{-23} J/K.

Negative values for the change in entropy are associated with lower degrees of freedom of molecular motions. This implies that upon the incorporation of TQ into HP β CD cavity, the change in entropy becomes negative as a result of the decrease in the molecular motion of TQ assuring its attempt in the penetration of HP β CD cavity and hence rendering the complexation system to seek stability and orderliness.

The inclusion of a bulky and rigid substituent group imposes a restriction on the arrangement of the cyclodextrin ring because the orientation of the substituent group is sterically restricted and the next cyclodextrin ring is arranged to include it.

ΔG may have negative or positive values depending on the temperature factor. If the temperature is low, then ΔG will have negative values, whereas if the temperature is high then ΔG will have positive ones. Accordingly, by carrying out the experiments under low temperatures in connection with the previously obtained values of ΔH and ΔS as appeared in Table 6.4, which are both negative, ΔG have been calculated to have negative values. This result suggests that the inclusion process of TQ into HP β CD cavity was spontaneous in nature.

3.1.9 Hydrodynamic size and zeta potential

The zeta potential for the TQ/HPBCD complex suspensions was -25.9 mV while the particle diameter was found to be 41.6 nm. Besides the biomolecular approach, it is well known that surface charge, shape, and size are the primary parameters of colloidal particles for cellular uptake (Al-Qubaisi et al., 2013b). Size analysis showed that the TQ/HPBCD inclusion has hydrodynamic diameters that are much larger than real size (0.6 to 0.65 nm) (Manasco et al., 2012). This may suggest that our complex tends to aggregate in deionized and double-distilled water, and their low zeta potential of -25.9 mV indicates that the formulated complex have poor electrostatic repulsion characteristics and thus are of incipient instability. At this zeta potential (-25.9 mV), the produced complex would not repel particle aggregation in suspensions for long-term stability. Generally, the cells have fewer uptakes of particles with negative charge compared to those with positive charge, which might be attributed to the attractive or repulsive interaction between the negatively charged cell membrane and positively/negatively charged particles (Al-Qubaisi et al., 2013b).

3.1.10 Scanning electron microscopy

TQ/HP β CD inclusion complex has regular and smooth surface (Figure 15). The SEM micrographs for HP β CD demonstrate the changes in habit of the crystals disappeared after complexation with TQ (Figure 15). The HP β CD powder has aggregated after the incorporation of TQ and the evaporation of solution, implying that the HP β CD has acted as a carrier, which covered the surface of TQ completely.

3.2 Anti-allergic activity

3.2.1 Cytotoxicity

The concentrations of pure TQ, TQ/HP β CD, and HP β CD screened for cytotoxicity on RBL 2H3 cells were tested (Figure 16). The compounds at all concentrations did not significantly ($P > 0.05$) inhibit cell growth therefore we choose them for anti-allergic studies.

3.2.2 Histamine release

A widely used in vitro model for hypersensitivity for mast cell functions is the rat basophil-type RBL-2H3 cell line. This cell line was derived from a rat basophilic leukemia and contains a gain-of-function mutation in the stem cell factor receptor c-kit 1, enabling continued proliferation of the cell line (Passante and Frankish, 2009).

Although the TQ has been shown to have diverse biological activities, including immune enhancing properties, anti-inflammatory antimicrobial, antifungal, anticancer, its role in allergic response is not clear. The effect of TQ and TQ/HP β CD inclusion complex on histamine release from RBL-2H3 cell line was determined to ascertain the potential of these in the alleviation of allergic responses. The effect of TQ, TQ/HP β CD inclusion complex and cetirizine on histamine release by RBL2H3 cells is shown in Figure 17. Histamine release by the RBL2H3 cells increased with decrease in TQ and TQ/HP β CD inclusion complex treatment concentrations. Treatment with 2.0 μ g/mL TQ dissolved in DMSO, TQ complexed with HP β CD, and cetirizine, caused decrease of histamine release by 58, 34, and 30%, respectively. Loading of TQ into inclusion complex system caused greater inhibition of histamine release than free TQ. At 1.0 μ g/mL of TQ loaded into inclusion complex, the inhibitory rate of histamine release by RBL2h3 cells was 62.1%, while cetirizine and free TQ showed 48.7 and 35.8% inhibition, respectively. The IC₅₀ of histamine release for TQ complexed with HP β CD and Cetirizine was 0.71 and 1.06 μ g/mL, respectively.

3.2.3 Interleukin-4

IL-4 release by RBL2H3 cells decreased dose-dependently by 1.29- and 1.52-fold after treatment with 0.125 and 0.5 μ g/mL of TQ complexed with HPBCD, respectively (Figure 18). TQ dissolved in DMSO began to inhibit IL-4 release at higher concentrations of 1.0 to 4.0 μ g/mL treatment dose. In cells treated with 4 μ g/mL TQ dissolved in DMSO and TQ complexed with HPBCD, the release levels of interleukin-4 were reduced to 2.29-, and 3.06-fold, respectively, compared to those exposed to media alone. IC₅₀ of IL-4 release for TQ dissolved in DMSO and complexed with HP β CD were 1.75 and 1.0 μ g/mL, respectively.

3.2.3 Tumor necrosis factor- α

TQ/HP β CD inclusion complex dose-dependent decreased in TNF- α release by RBL2H3 cells, that is progressively greater decrease with increasing treatment concentrations (Figure 19). This effect is more prominent than that seen with TQ dissolved in DMSO at the same concentrations. IC₅₀ of IL-4 release for TQ dissolved in DMSO and complexed with HP β CD were 1.62 and 0.17 μ g/mL, respectively. Thus, it is evident that incorporation of TQ HP β CD had increased anti-allergy efficacy of TQ.

4.4. Conclusion

Complexation with HP β CD had improved solubility of the poorly water-soluble TQ. The release of TQ followed the Weibull model release kinetics, and is faster in the very acid medium of the gastric than the lesser acidic intestinal juice. The encapsulation of TQ in TQ/HP β CD inclusion complex also improved its thermal stability. Thus, the TQ-HP β CD has all features and advantages of sustained-release drug carrier system. TQ-HP β CD is also more efficacious as an anti-allergy compound than either TQ or the anti-allergy drug, cetirizen. Therefore, our future studies will focus on providing additional pharmacological evidence to demonstrate this possibility.

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Figures

Figure 1: Structure of thymoquinone

Figure 2: Powder X-ray diffraction patterns for thymoquinone (TQ), TQ-loaded hydroxypropyl- β -cyclodextrin (TQ-HP β CD), physical mixture, and hydroxypropyl- β -cyclodextrin (HP β CD).

Figure 3: Fourier transform infrared spectrum for thymoquinone (TQ), hydroxypropyl- β -cyclodextrin (HP β CD), physical mixture, and TQ/HP β CD inclusion complex (TQ-HP β CD).

Figure 4: Differential scanning calorimetric curves for thymoquinone (TQ), TQ/HP β CD inclusion complex (TQ-HP β CD), physical mixture, and hydroxypropyl- β -cyclodextrin (HP β CD).

Figure 5: TGA-DTG thermograms for thymoquinone (TQ), hydroxypropyl- β -cyclodextrin (HP β CD), and TQ/HP β CD inclusion complex (TQ-HP β CD).

Figure 6: The ^1H NMR Spectra for (a) thymoquinone (TQ), (b) hydroxypropyl- β -cyclodextrin (HP β CD) and (c) thymoquinone-loaded HP β CD (TQ-HP β CD). Recorded in deuterated methanol at 25 °C.

Figure 7: The interaction between guest thymoquinone (TQ) and host hydroxypropyl-beta-cyclodextrin (HP β CD).

Figure 8: 2D ^1H - ^1H COSY spectrum for thymoquinone-loaded hydroxylpropyl- β -cyclodextrin (TQ-HP β CD) in D_2O at 25 °C.

Figure 9: 2D NMR NOESY spectrum for thymoquinone-loaded hydroxypropyl- β -cyclodextrin (TQ-HP β CD).

Figure 10: Release of thymoquinone from complex and physical mixture in artificial intestinal juice (AIJ) and artificial gastric juice (AGJ).

Figure 11: Kinetic models of thymoquinone release from thymoquinone-loaded hydroxypropyl- β -cyclodextrin (TQ/HP β CD) in artificial gastric juice. OBS and PRED are the observed and predicted values respectively.

Figure 12: Kinetic models of thymoquinone release from thymoquinone-loaded hydroxypropyl- β -cyclodextrin (TQ/HP β CD) in artificial intestinal juice. OBS and PRED are the observed and predicted values respectively.

Figure 13: Phase solubility of thymoquinone (TQ) in the presence of hydroxypropyl- β -cyclodextrin (HP β CD) at 293 K, 303 K, 310 K, and 318 K.

Figure 14: The relationship between $\ln K$ and $1/T$ in the interaction between thymoquinone and hydroxylpropyl- β -cyclodextrin.

Figure 15: Morphology of HP β CD and TQ/HP β CD inclusion complex (TQ-HP β CD).

Figure 16: Pure TQ, TQ/HP β CD inclusion complex, and HP β CD effects on the viability of treated cells, which were evaluated through mitochondrial activity using MTT assay on RBL 2H3 cells 24 hours. Mean $\pm$ SD ($n = 3$ wells/treatment). The concentration refers to TQ either free (dissolved in DMSO) or complexed with HP β CD.

Figure 17: Histamine release from RBL2H3 cells treated with TQ, TQ/HP β CD inclusion complex, and Cetirizine. The concentration refers to TQ either free (dissolved in DMSO) or

complexed with HP β CD. Mean $\pm$ SD (n = 3 wells/treatment). *P < 0.05 compared with the untreated cells.

Figure 18: Interleukin-4 (IL-4) release from RBL2H3 cells treated with TQ and TQ/HP β CD inclusion complex. The concentration refers to TQ either free (dissolved in DMSO) or complexed with HP β CD. Mean $\pm$ SD (n = 3 wells/treatment). *P < 0.05 compared with the untreated cells.

Figure 19: Tumor necrosis factor- α (TNF- α) release from release from RBL2H3 cells treated with TQ and TQ/HP β CD inclusion complex. The concentration refers to TQ either free (dissolved in DMSO) or complexed with HP β CD. Mean $\pm$ SD (n = 3 wells/treatment). *P < 0.05 compared with the untreated cells.

Tables

Table 1: The chemical shifts (δ) of protons of the hydroxypropyl- β -cyclodextrin (HP β CD), thymoquinone (TQ) and thymoquinone-loaded HP β CD (TQ-HP β CD).

Table 2: Data-fitting for thymoquinone release from thymoquinone-loaded hydroxylpropyl- β -cyclodextrin (TQ/HP β CD) in artificial gastric juice.

Table 3: Data-fitting for thymoquinone release from thymoquinone-loaded hydroxylpropyl- β -cyclodextrin (TQ/HP β CD) in artificial intestinal juice.

Table 4: The effect of temperature on K, Δ H, Δ S and Δ G of the inclusion complex of TQ-HP β CD.

Table 1

Proton	HP β CD (δ)	TQ (δ)	TQ- HP β CD (δ)	Δ (δ)
H1	5.131	-	5.119	- 0.012
H2	3.546	-	3.542	- 0.004
H3	3.960	-	3.962	0.002
H4	3.428	-	3.427	- 0.001
H5	3.738	-	3.739	0.001
H6	3.835	-	3.836	0.001
Ha	-	1.109	1.141	0.032
Hb	-	1.990	2.012	0.022
Hc	-	2.963	2.997	0.034
Hd	-	6.560	6.540	- 0.020

Table 2

Model	Slope	Intercept	R^2	R^2_{emp}	RMSE	AIC	BIC	k
Zero-order	0.036000	29.300000	0.79700	0.79721	13.1000	32500.0	32500.0	-
First-order	0.000781	3.160000	0.40600	0.21167	25.9000	35900.0	35900.0	-
Second-order	-9580.000000	16000000.000000	0.00120	-6.51315	79.9000	41500.0	41500.0	-
Korsmeyer-Peppas	0.732850	-0.855470	0.91912	0.78114	13.6350	32655.4	32667.1	0.425
Weibull	1.048340	-6.776320	0.94076	0.97899	4.2248	26792.4	26804.0	876.837
Hixson-Crowell	0.000879	2.980410	0.68381	0.5952	18.5438	34194.1	34205.8	0.001
Higuchi	0.500000	0.731250	0.87042	0.87042	10.4916	31344.0	31355.7	2.078
Baker-Lonsdale	0.000205	-0.021210	0.94577	-6.51315	79.8892	41502.5	41514.1	-
Michaelis-Menten	1.000000	0.019520	1.00000	-0.59585	36.8191	37626.3	37637.9	51.231

MDT: 627.907

DE: 74.3927

No of timepoints: 2502

Table 3

Model	Slope	Intercept	R²	R²_{emp}	RMSE	AIC	BIC	k
Zero-order	0.005165	52.663219	0.75085	0.75085	8.5913	135166	135180	-
First-order	0.000087	3.883379	0.30155	0.60508	10.8164	139774	139788	-
Second-order	-599.580224	3998200.805026	0.00030	-20.79716	80.3582	179894	179909	-
Korsmeyer-Peppas	0.385096	1.155254	0.78249	0.75020	8.6024	135192	135206	3.1748
Weibull	0.638081	-4.749663	0.87601	0.93218	4.4823	122150	122164	115.5454
Hixson-Crowell	0.000109	3.701937	0.56800	0.69253	9.5441	137270	137284	0.0001
Higuchi	0.500000	0.212125	0.24633	0.24633	14.9424	146238	146253	1.2363
Baker-Lonsdale	0.000035	0.036014	0.94172	-20.79716	80.3582	179894	179909	-
Michaelis-Menten	1.000000	0.014111	1.00000	0.05728	16.7118	148477	148492	70.8669

MDT: 2150.8

DE: 78.5

No of timepoints: 10003

Table 4

T	1/T	K	lnK	ΔS (J mol ⁻¹ K ⁻¹)	ΔH (kJ mol ⁻¹)	ΔG (kJ mol ⁻¹)
293	0.0034	9348.7	9.143	-45.9	-36	-22.5
303	0.0033	5450.7	8.6035	-45.9	-36	-22.1
310	0.0032	3814.5	8.2466	-45.9	-36	-21.8
318	0.0031	2586.1	7.8579	-45.9	-36	-21.4

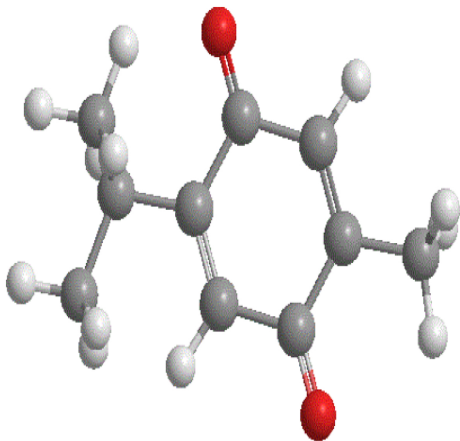


Figure 1

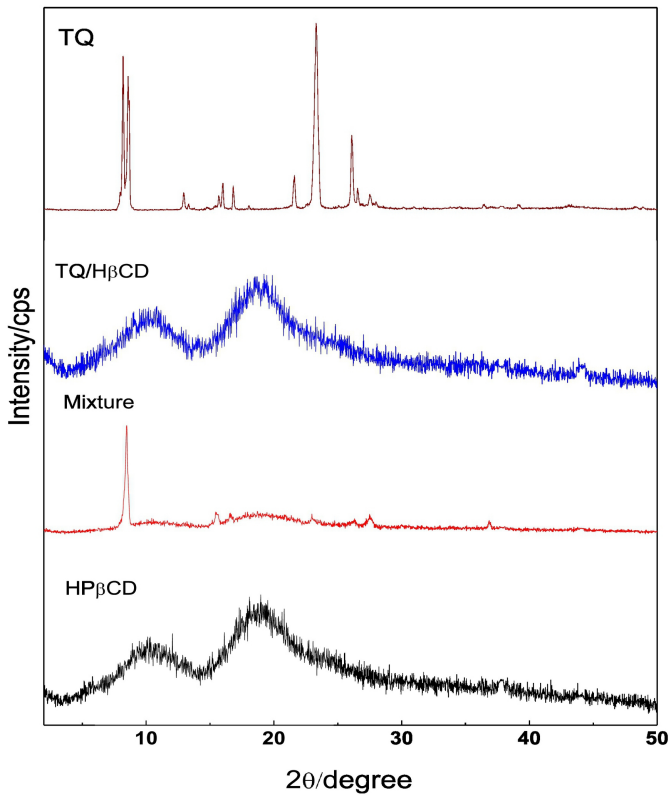


Figure 2

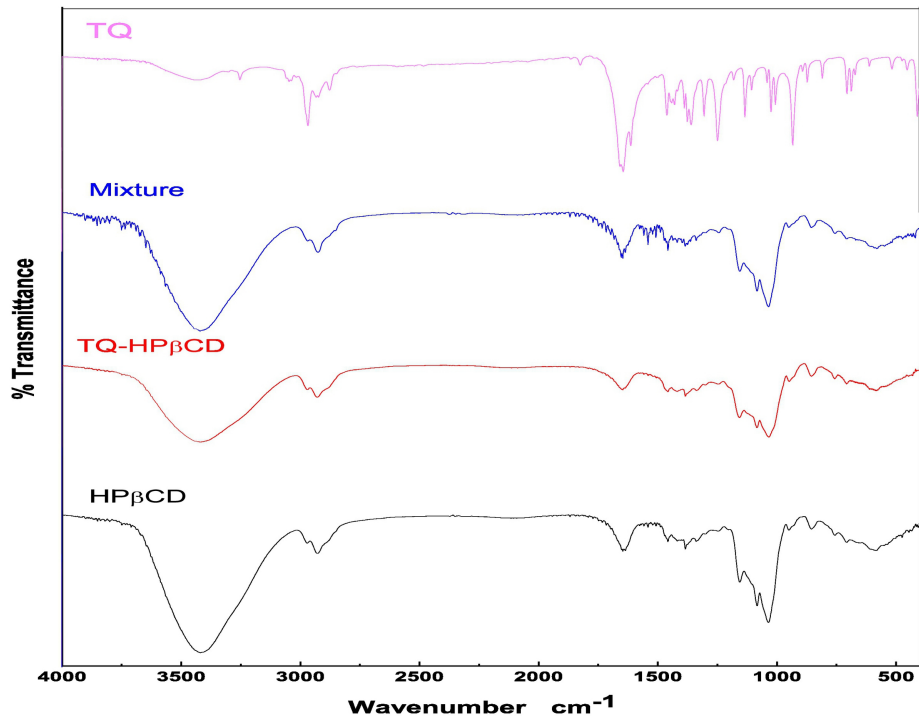


Figure 3

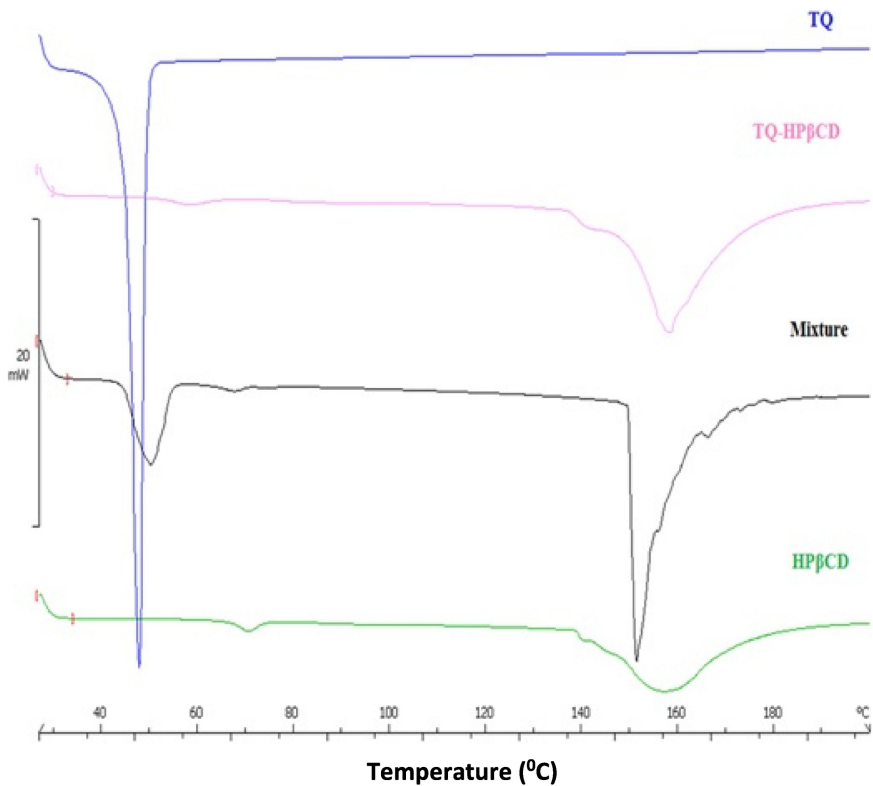


Figure 4

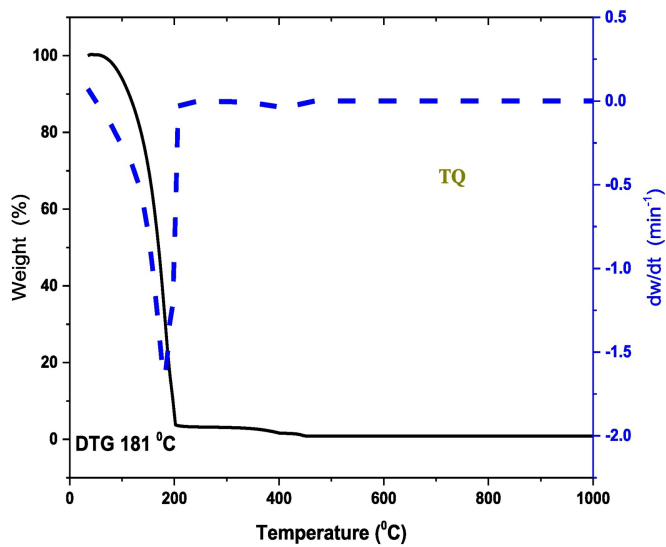
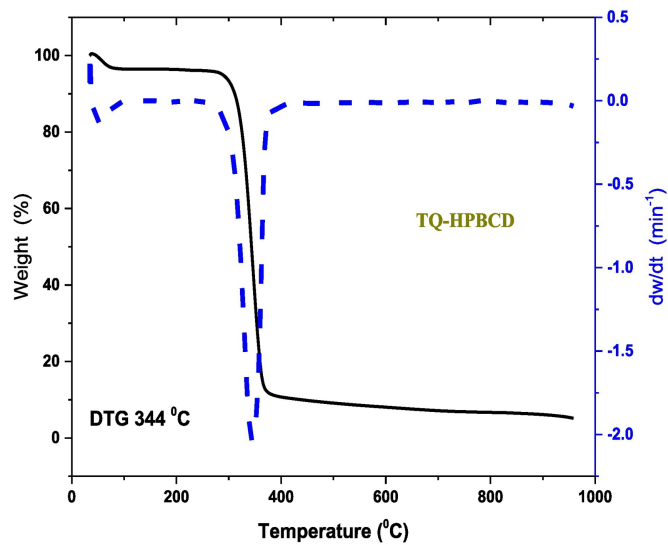
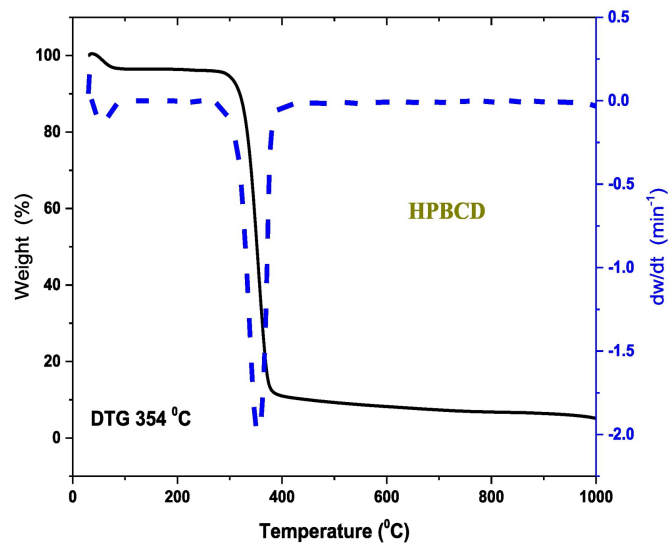


Figure 5

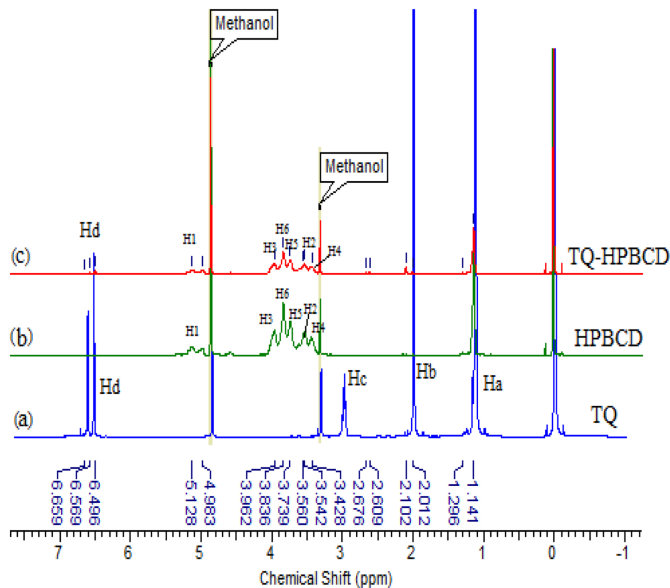
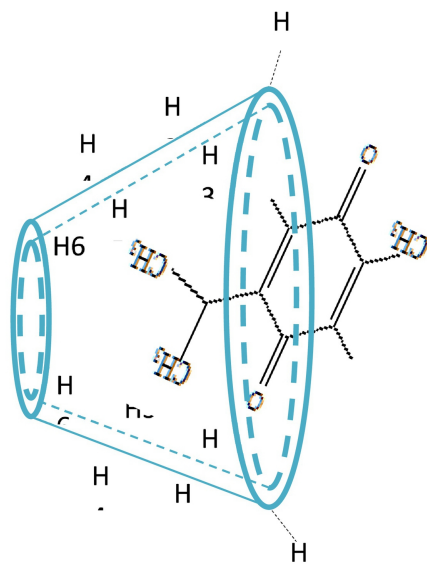
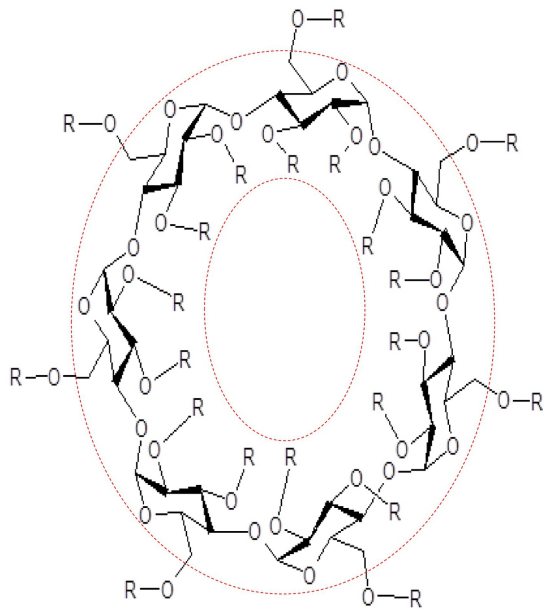
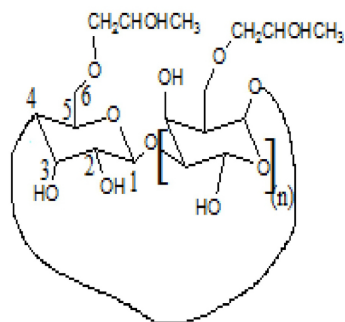


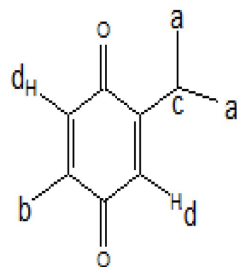
Figure 6



R= -



HPBCD, $n=6$.



Thymoquinone (TQ)

Figure 7

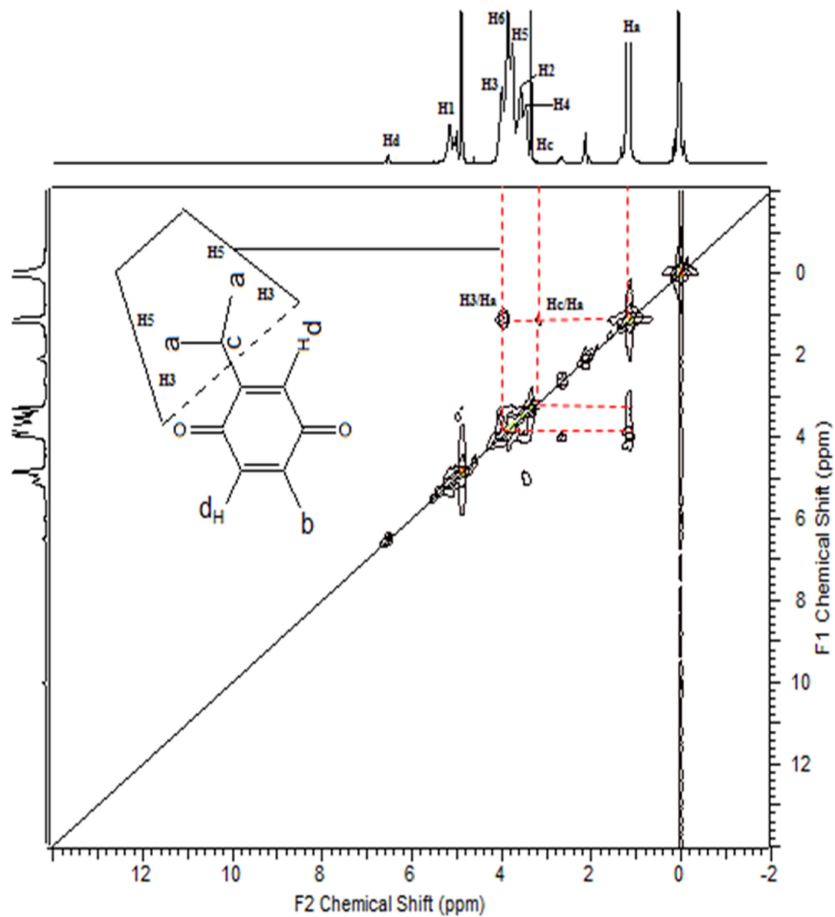


Figure 8

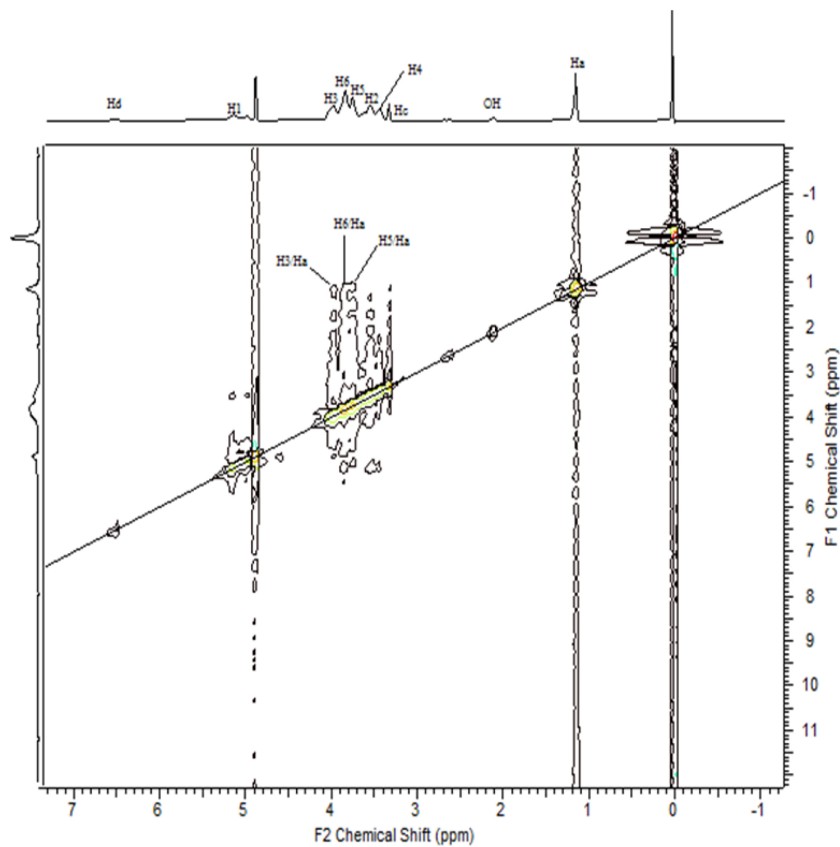


Figure 9

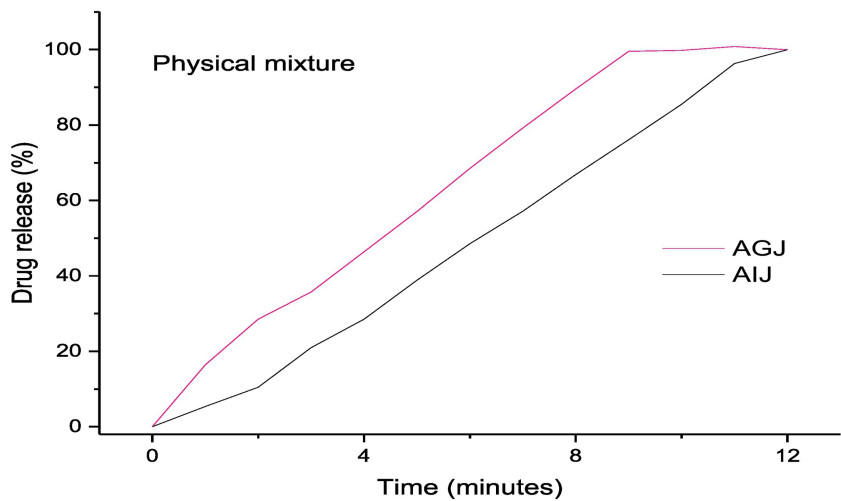
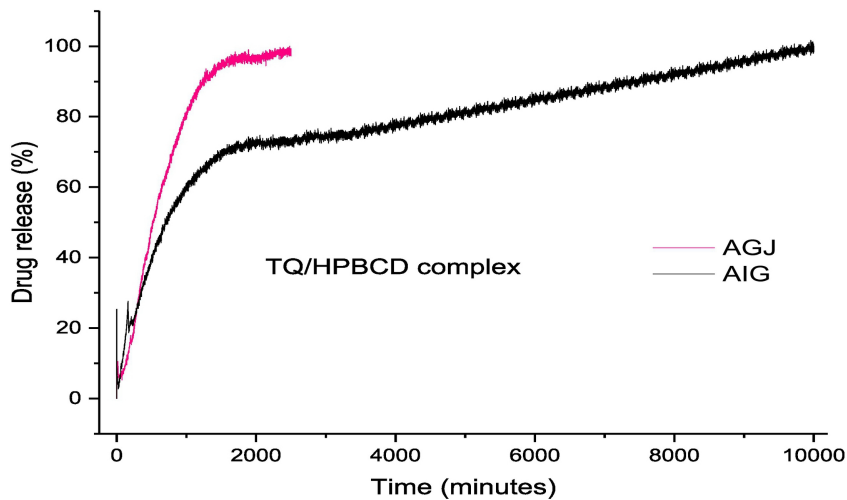


Figure 10

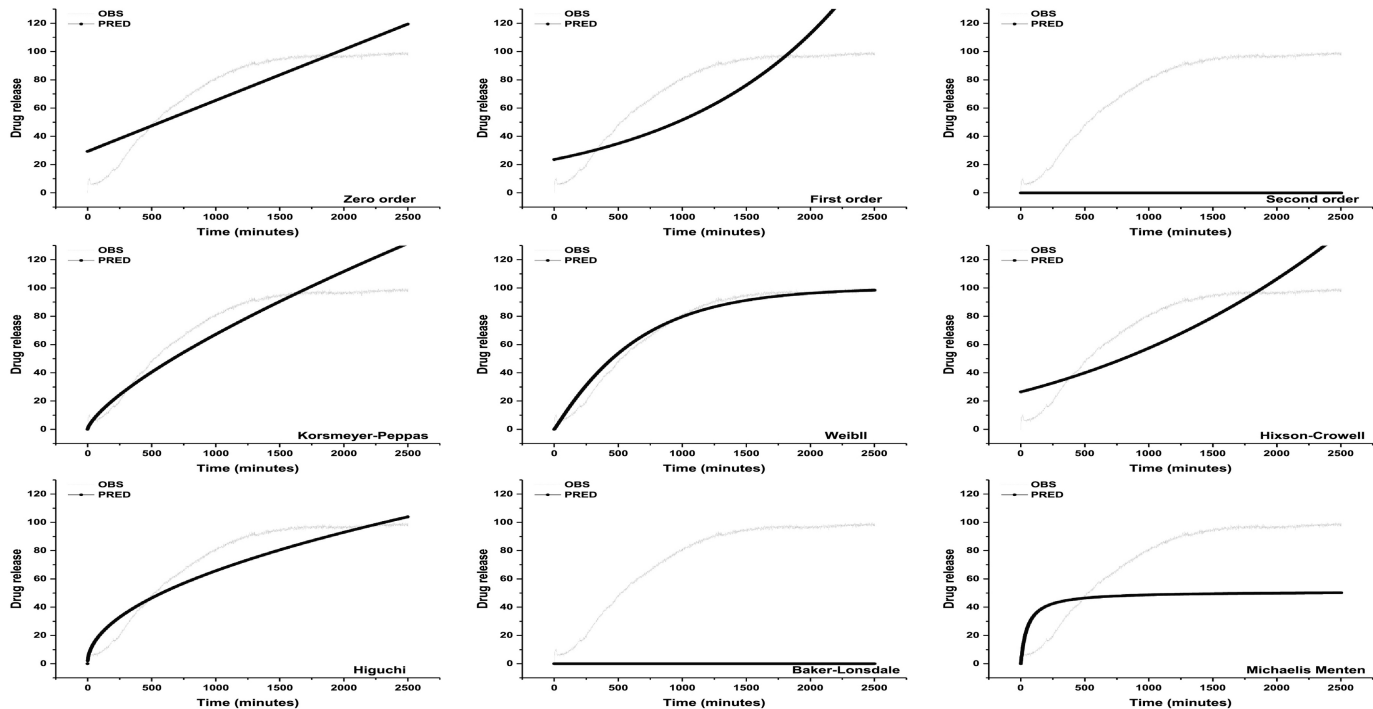


Figure 11

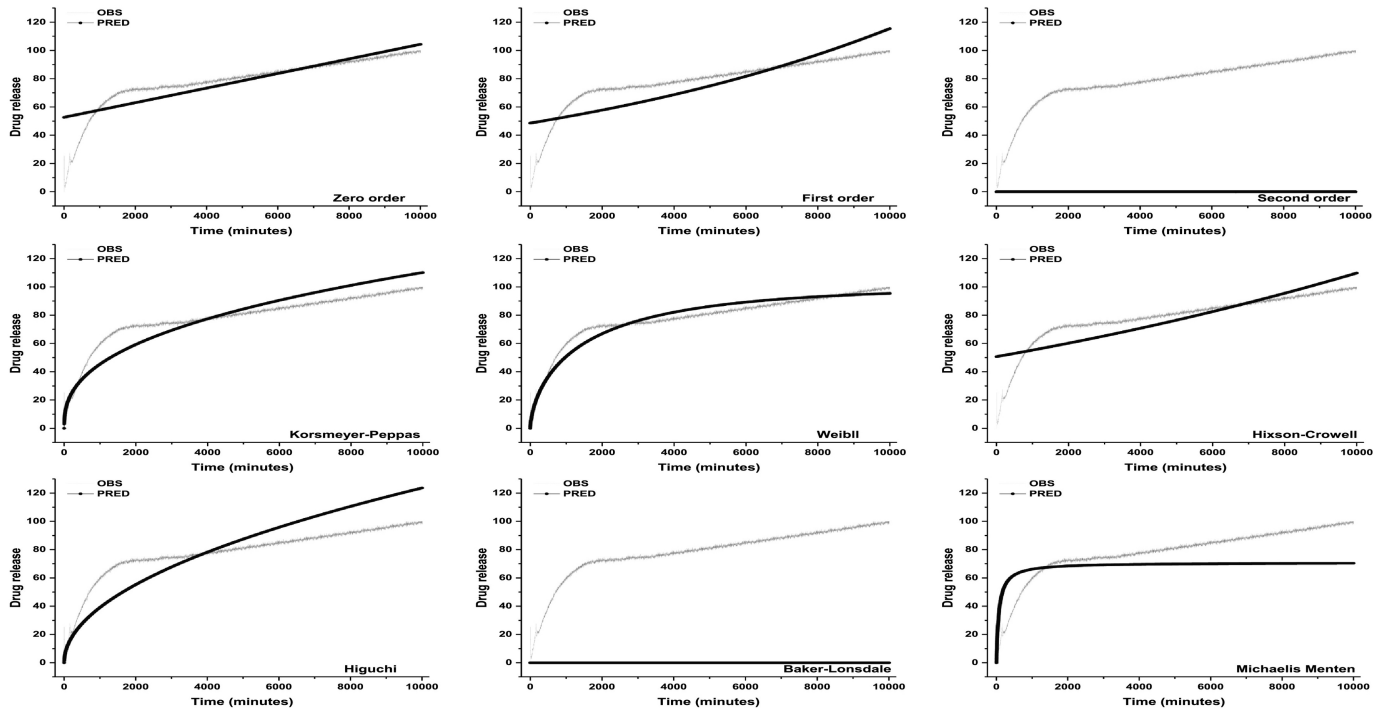


Figure 12

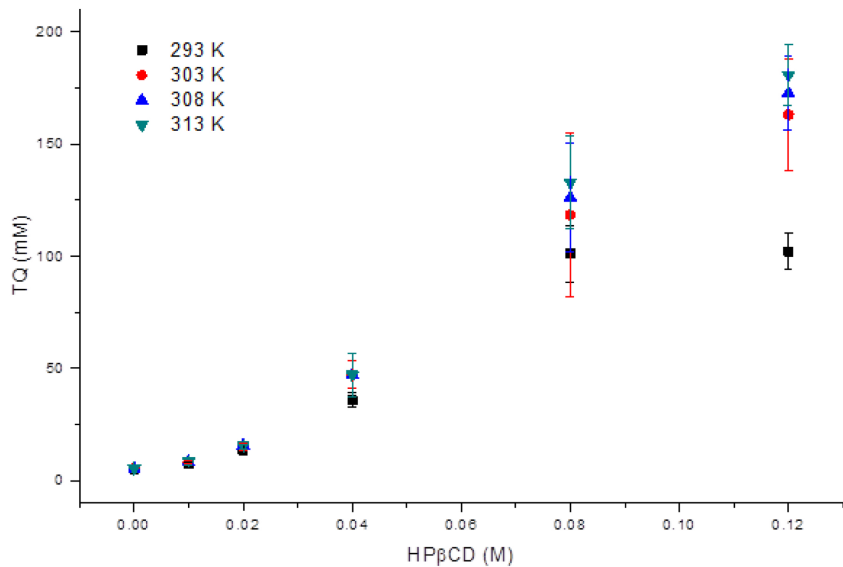


Figure 13

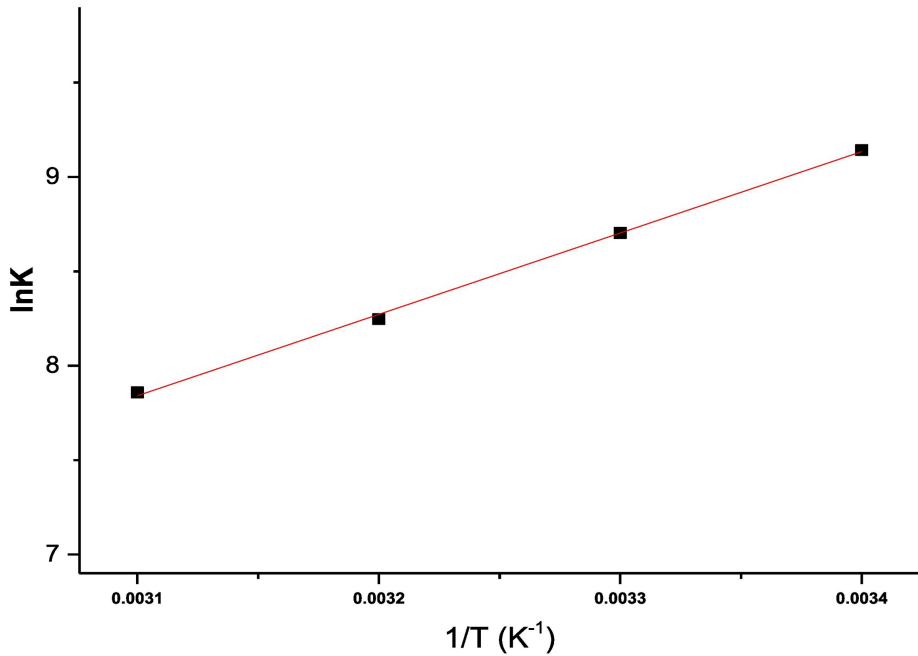


Figure 14

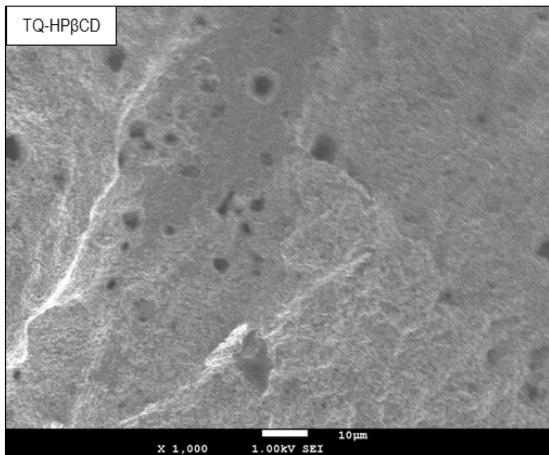
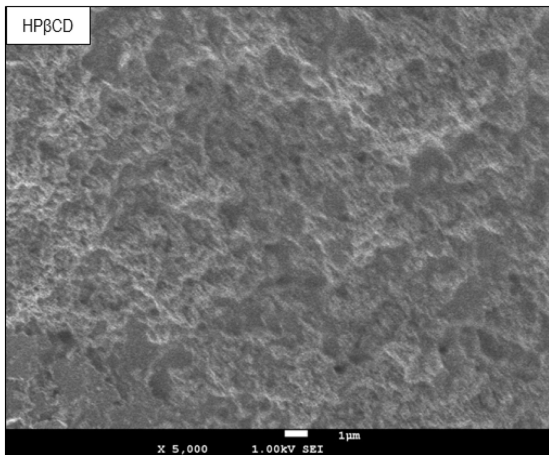


Figure 15

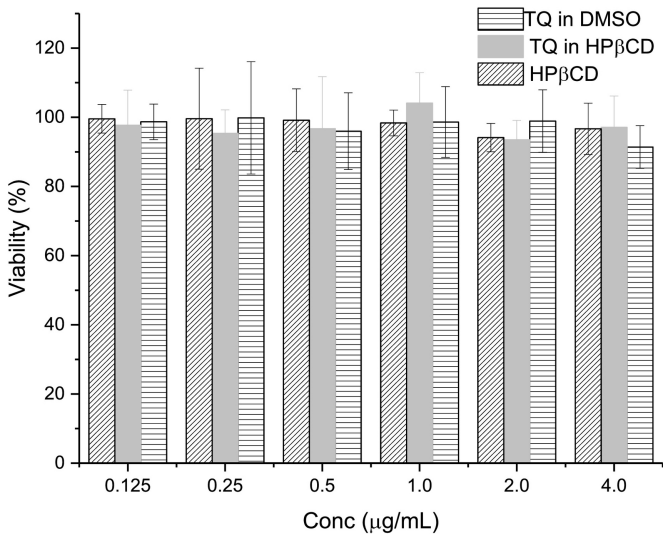


Figure 16

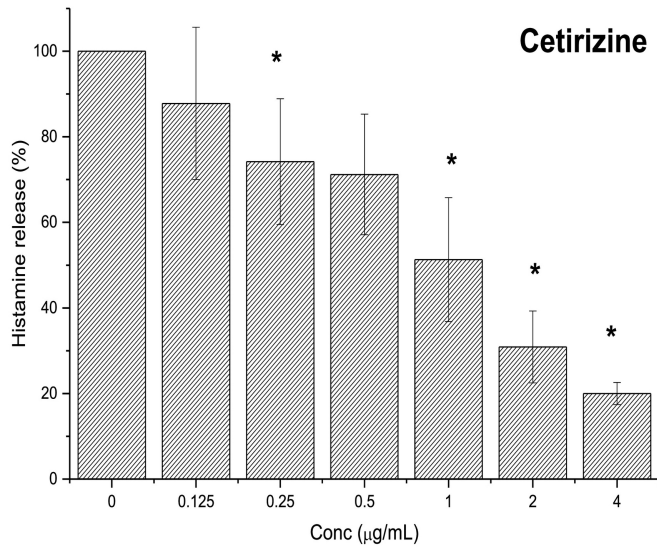
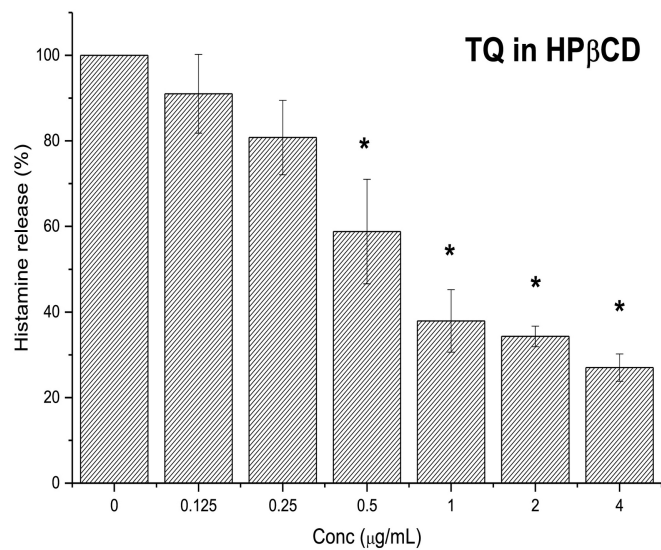
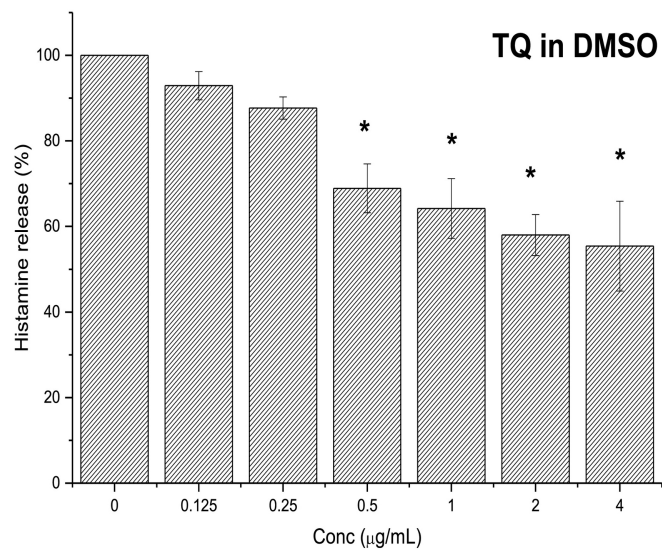


Figure 17

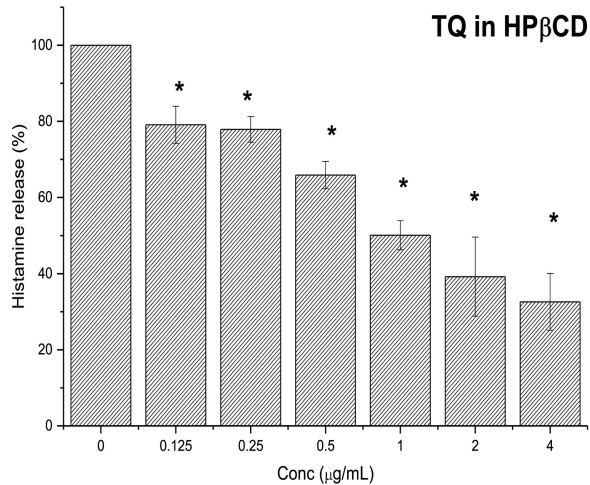
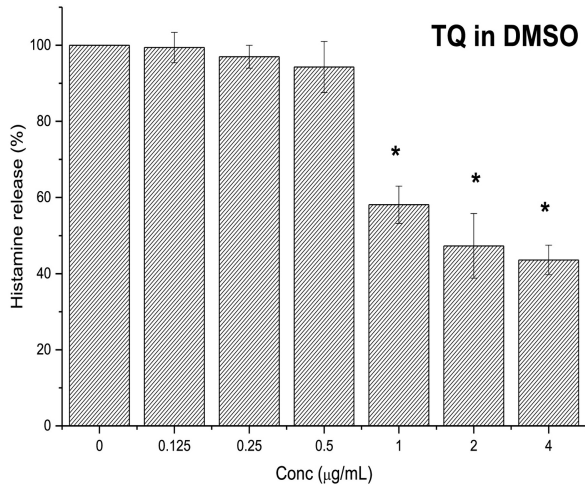


Figure 18

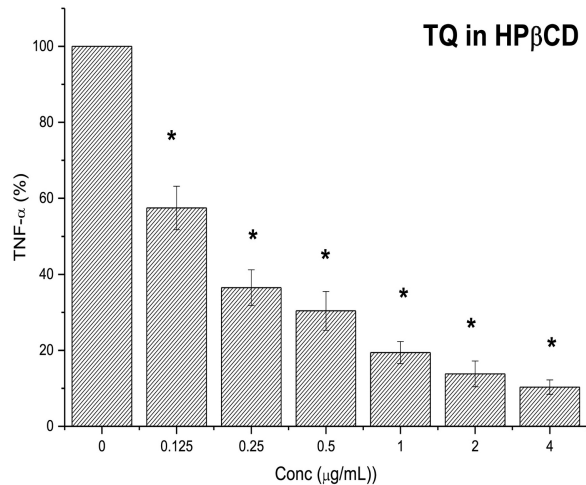
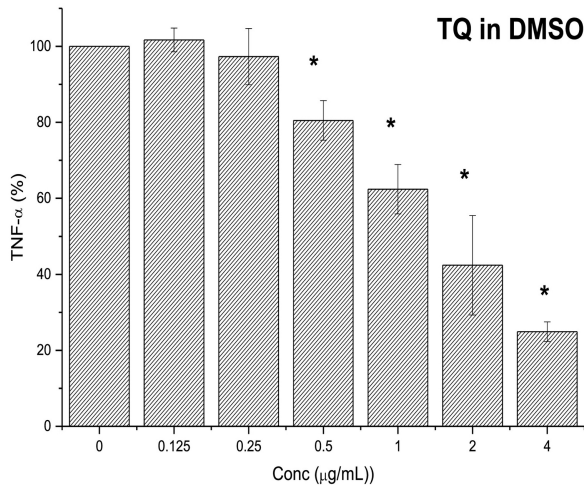


Figure 19